A STUDY OF THE ANTIGENIC STRUCTURE OF THE PLAGUE BACILLUS (PASTEURELLA PESTIS EV NIIEG) WITH THE AID OF DIFFUSION PRECIPITATION IN AGAR

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/Following is a translation of the Russian language article by V. L. Pustovalov, N. G. Ovanesova, A. M. Konnova and L. I. Kolesnikova of the Rostov on the Don Antiplague Institute. It appeared in the journal "Particularly Dangerous and Naturally Focal Infections" which was published by the State Publishing House for Medical Literature, Moscow, 1962. Translation performed by Sp/6 Charles T. Ostertag Jr./ (Pp 140-149)

The study of the antigenic structure of the plague bacillus is of great interest from the point of view of the subsequent isolation of these substances and the study of their chemical composition and immunogenic properties.

The development by Oudin (1948) and Ouchterlony (1949) of the method of diffusion precipitation in gel was an important step in the analysis of the antigenic structure of microorganisms (Yu. Z. Gendon, 1958).

The gel diffusion precipitin technique was used in subsequent years for studying the antigens of the plague bacillus.

Ransom et. al. (1955) investigated the antigenic structure of five various strains of plague bacillus and obtained 10 precipitation zones.

Chen and Meyer (1955) studied the antigenic structure of a number of strains of the plague bacillus and fractions from them, and also one strain of the pseudotuberculosis microbe. It was found that the avirulent, but antigenic, strain A1122 contained fraction I of Baker et al. (1947, 1952), and the avirulent, but nonantigenic, strains 14 and TRU were almost entirely lacking in fraction I of Baker.

Bhagavan, Chen, and Meyer (1956) established 7 antigens in the composition of virulent and avirulent strains of the plague bacillus. Five of them turned out to be common with antigens of the pseudotuberculosis microbe.

Crumpton and Davies (1956) exposed 10 antigens in the avirulent Tjiwidej strain. A line of precipitation was identified which corresponded to the envelope antigen and also lines corresponding to plague toxin and an antigen, the specificity of which was determined by a polysaccharide component.

Burrows and Bacon (1956) observed 12 - 18 lines of precipitation when setting up the reaction with live plague microorganisms. In

the composition of all the virulent strains and also the EV 76 strain they detected the V and W antigens which depress phagocytosis.

S. I. Zaplatina (1958) investigated the antigenic structure of a number of fractions from a virulent (177) and avirulent (EV 76) strain of the plague bacillus, and also the pseudotuberculosis microbe (strain 498). In another work, S. I. Zaplatina (1957) reported the presence in live plague cultures of various origin of 7 antigens, 4 of which were common with the antigens of pseudotuberculosis cultures. V. V. Akimovich and T. Ye. Obrotsvetova (1960) established that virulent and toxic strains of plague bacillus have as a minimum 5 antigenic elements.

Lawton et al. (1960) found 16 antigens in the composition of the plague bacillus and 13 antigens in the composition of the pseudotuberculosis microbe; 11 antigens were common for both species. Among the common antigens, an L-antigen was discovered which the authors consider immunogenic.

In the present work we have undertaken an attempt with the aid of diffusion precipitation in agar to determine the overall number of antigens in the avirulent strain of P. pestis EV (NIIEG), and also to follow their diffusion during the fractionation of substances extracted from a bacterial mass.

In this work we used the avirulent EV (NIIEG) strain of P. pestis which was incubated for three days at 37° on casein-hydrolysate agar (pH 7.2) with a 5% solution of yeast autolysate (Ye. M. Gubarev, S. I. Zaplatina, A. M. Konnova, 1956). The bacteria were washed from the surface of the agar with a physiological solution containing an 0.001M phosphate buffer (pH 7.1) and killed by the addition to the suspension of 2 volumes of acetone cooled to -35°. After two days storage in a refrigerator (2°) the bacterial mass was separated out by centrifugation, then rinsed on a filter with cold acetone and dried in a vacuum (5-10 mm mercury column).

With the addition of 3 more volumes of acetone to the acetone-aqueous centrifugate it yielded a precipitate which was collected, dialyzed and dried by lyophilization. This substance, which is well soluble in a physiological solution and distilled water, was named precipitate R.

The scheme suggested by Baker et al. (1952) of using ammonium sulfate for the extraction and fractionation of plague bacillus antigens for the further isolation of IA, IB, and the toxic fractions was used by us for studying the distribution of antigens in these fractions. With this aim 129.2 grams of an air dried bacterial mass was subjected to three successive extractions with a 2.5% solution of NaCl (pH 7.1). By a method of infusion (with a periodic shaking),

three extracts were obtained in the course of 24 hours. The three extracts were separated out from the bacterial mass by centrifugation (2^{o}) at 3,000 rpm for two hours. The first extract (E_1) had a volume of 1370 ml, the second (E_2) -- 1405 ml, and the third (E_3) -- 1040 ml. A calculation of the dry residue in samples from each extract showed that the content of dry substance minus the content of NaCl in E_1 extract was 17.9 mg/ml, in E_2 extract -- 6.4 mg/ml, and in E_3 extract -- 1 mg/ml. Then all the extracts were combined. As a result, 3,815 ml of combined extract was obtained which by calculation contained 32.3 grams of substance (25.0% in relation to the initial bacterial mass). The substances found in the combined extract were subjected to fractionation with ammonium sulfate (figure 1). All the fractions were dialyzed free of ammonium sulfate and dried by lyophilization.

As a result of the addition of up to a 25% saturation of ammonium sulfate, fraction alpha$_1$ was isolated. It contained a large amount of agar from the nutrient medium.

With a 30% saturation of ammonium sulfate an unpurified fraction IA was precipitated which then was reprecipitated several times in order to purify it from the admixture of agar (fractions a_2, a_3, a_4)* and the admixture of other antigens (fractions Ts_1, Ts_2, and Ts_3). Purified fraction IA (precipitate IA4) has a protein-polysaccharide composition, giving a positive Biuret reaction and a positive Molisch test.

*(Here the original text uses the Cyrillic letter а, but evidently meant the Greek letter alpha.)

With a 40% saturation of ammonium sulfate an unpurified fraction IB was isolated which was then reprecipitated and purified from fraction IA (fractions K, L, M)* and from the toxic fraction (Fractions gamma$_1$, gamma$_2$, and gamma$_3$). The purified fraction IB (precipitate IB4) is a protein substance without the admixture of a polysaccharide (positive Biuret reaction and negative Molisch test).

*(In figure 1 fraction K is listed as a residue while fractions L and M are listed as precipitates.)

With a 67% saturation with ammonium sulfate the toxic fraction was precipitated. The substance left in the centrifuge was dialyzed, lyophilized and designated as the residue fraction.

The quantitative yield and chemical characteristics of the fractions are presented in the following table.

Properties of fractions isolated from an extract of P. pestis EV (NIIEG)

Name of fraction	Weight, g.	Nitrogen content, %	Phosphorus content, %
$alpha_1$	0.1876	-	0.03
$alpha_2$	0.5020	8.10	0.03
$alpha_3$	1.3323	9.73	0.01
$alpha_4$	0.0232	-	-
Ts_1	0.4220	10.42	0.11
Ts_2	0.5624	11.09	0.03
Ts_3	0.0301	-	-
IA_4	0.9006	10.58	0.05
IB_4	0.2280	14.03	0.03
K	0.7094	9.77	0.03
L	0.0340	-	-
M	0.0283	-	-
$gamma_1$	0.7890	8.70	0.01
$gamma_3$	0.0465	8.50	0.03
toxic	5.2095	-	0.16
residue	2.9980	-	0.19
R	2.9343	-	0.15

The extracts from the bacterial mass (E_1, E_2, and E_3), the precipitate R, and also all the isolated fractions were investigated by Ouchterlony's method of diffusion precipitation in agar (1949) with the aim of ascertaining the total number and distribution of antigens in the plague bacillus.

In the capacity of gel we used 1% agar-agar in an aqueous solution containing 0.85% NaCl, 0.25% phenol and an 0.001M phosphate buffer (pH 7.2). Into each Petri dish 40 ml of hot agar solution was poured. After the gel had solidified, receptacles 15 or 18 mm in diameter were made in the agar plate with a sharp cylindrical punch. Two drops of a molten agar solution were added into the bottom of each receptacle for sealing. The receptacles were laid out by using a template which conformed to the conditions of the experiment. The distance between the receptacles was 15 or 20 mm. The experiments based on precipitation in gel were set up in two ways. In the first or A method, batches of material were diluted in an 0.5 ml physiological solution and in one operation were placed into the appropriate receptacles. Simultaneously 0.5 ml of plague agglutinating serum was added to the corresponding hole. When receptacles with a diameter of 18 mm were used the batches of material were diluted in 1 ml and each solution was placed in the appropriate receptacle in one operation. In this case 1 ml of serum is placed in the specific hole. In this

work we used serum from the Saratov "Mikrob" Institute (series 66 and 68, titer 1:2,000) which was obtained by the immunization of horses with mixtures of cultures of P. pestis 1, 17 and EV. The dishes were covered with covers which had affixed to their inner surface discs of filter paper with a diameter equal to the dimensions of the dish. The dishes were placed in a horizontal position in an incubator at 37°. The filter paper in the covers of the dishes was moistened with distilled water every day. In the second or B method, batches of material were diluted in 1 ml of a physiological solution and placed in the appropriate receptacles (15 mm diameter) over a period of 4 days. In this, on the first day 0.5 ml of the solution was placed into a receptacle and the remaining amount added up to the edge during the next three days. Serum was added into the appropriate receptacle in precisely the same way over a period of four days. During the next 14 days, physiological solution (pH 7.1) was placed in all the receptacles. The dishes were covered with covers which had discs of filter paper 4 cm in diameter affixed to the inner surface with leukoplaster and placed in an incubator at 37°. The filter paper discs were moistened with distilled water every day. We used the B method described for a more effective separation of the precipitation lines. These lines are formed more gradually and with the addition of the physiological solution are drawn aside from each other somewhat, apparently because of the nonuniform level of ultrafiltration of the particles of the precipitate through the gel. The final calculation of the reaction was conducted on the 20th day of the experiment.

In a number of cases for the more precise interpretation of antigenic structure we used plague agglutinating serum, absorbed by fraction IA. For preparing it we added 25 mg of fraction IA to 10 ml of serum. The mixture was thoroughly pulverized in a test tube with a glass rod and incubated at 37° for three hours with a periodic agitation. After this the precipitate was centrifuged at 16,000 rpm and a temperature of 2°. The absorbed serum was stored in a refrigerator until the moment of use. Such serum didn't produce a precipitation zone when checked by the method of precipitation in agar against fractions IA and IB.

Special experiments, using as a control of the physiological solution the extract from a sterile nutrient medium for cultivation of the plague microbe, and also normal horse serum, didn't show any kind of precipitation zones over a monthly observation period.

It is necessary to note that a comparison of the antigenic structure of live bacteria (P. pestis EV NIIEG), taken in a quantity of 75 billion in a receptacle (in an 0.5 ml volume of physiological solution) with a suspension of the same amount of these microorganisms that have been acetone killed showed a very similar pattern of precipitation lines (fig. 2).

In figure 2 the precipitation zones, corresponding to fraction I of Baker, are found near the receptacle with the microorganisms since the number of bacteria taken is too small for creating these zones in the place where they are usually localized.

A study of the antigenic structure of extracts E_1, E_2, and E_3 showed that they contained a large number of antigens (fig 3). With the aim of systematization we designated these precipitation bands with letters of the Russian alphabet, beginning from the receptacle with the solution of antigens and in the direction of the receptacle with the serum. Changes of concentration of the substances in the extracts from E_1 to E_3 made it possible to expose a number of antigens which have fusing precipitation bands at high concentrations. Thus, the thickest precipitation band (P) in extracts E_1 and E_2, which corresponds to the fraction I of Baker et al. (1952), in extract E_3 is already clearly separating into 4 bands. A study of the behavior of this band in figure 4, and also on a great number of other dishes, permits the supposition of the presence in it of substances which when the experiments are set up by method B (see fig 3) produce up to 6 precipitation bands. Thus the heterogeneity to fraction I of Baker et al. is made apparent.

From the external side of the described band (see fig 3) in the direction of the receptacle with E_1 there is a wide precipitation band which at E_2 separates into 3 zones (MNO). Two closely lying bands ZI are found yet further toward the outside, and more toward the periphery a wide band is found which at E_2 is separated into zones ZhYe and DG; after these follow bands V, B and right at the receptacle with the solution of antigens - band A.

Inwards from the thick line P there is yet one more wide precipitation band consisting of the 2 components UF. Between this band and the fraction I band is a small zone T. Inwards from the UF band in the direction of the hole with the serum, three more zones are noticed -- Kh, Ts, and Ch.

It must be noted that the thick precipitation line P contains two more precipitation zones RS, which by their position coincide with it. Setting up the precipitation reaction with plague agglutinating serum absorbed by fraction IA makes it possible to clearly expose the presence of zones RS (fig 5). In figure 6, bands K and L are also clearly visible. They lie between zones ZI and MNO.

Between the receptacle with E_2 extract and the receptacle with serum, 16 precipitation bands (see fig 3) are immediately observed. However, keeping in mind the expressed consideration about the complexity of band P and the presence of several more zones which are apparent in figures 5 and 6, the presence can be affirmed in the extract from P. pestis EV (MIIMO) of antigens which produce 28 precipitation zones. Figure 7 depicts a schematic of the antigenic structure of P. pestis EV (MIIMO).

In the process of work we made a number of extractions of various portions of a bacterial mass of P. pestis EV (NIIEG) and with these extractions a large number of reactions were set up of double diffusion precipitation in agar. A study of the precipitation pictures completely supported the proposed arrangement of the antigenic structure of P. pestis EV (NIIEG) incubated on a medium of caseinic acid hydrolysate.

It is necessary to note that the very intensive precipitation band P may sometimes inhibit the diffusion through it of antigens T, UF, and KhTsCh. In figure 8 it is apparent that a decrease in the receptacles of the amount of extract of the bacterial mass leads to the appearance of zones UF, Kh and Ts.

Investigation by the method of precipitation in agar of the fractions of the antigens of P. pestis EV (NIIEG) showed that they were heterogenous according to their composition. Fractions IA4, IB4, $alpha_2$, $alpha_3$, and $gamma_3$ produce the precipitation band P without the admixture of other zones. It must be noted during this that by the method of precipitation in agar, fractions IA and IB cannot be distinguished from each other. Sera adsorbed either by fraction IA or fraction IB equally do not produce precipitation lines corresponding to fraction I of Baker with the bacterial extract. With a number of special experiments it was also established that serum absorbed by fraction IA doesn't produce a precipitation line with a substance of fraction IB and vice versa.

Fractions $alpha_1$, Ts_1, Ts_2, K and $gamma_1$ produce precipitation band P accompanied by one of several other zones which testifies to the contamination of fraction I in these preparations with other antigens.

Precipitation in agar of the toxic and residue fractions is presented in figure 9. It is evident that in toxic just as in the residue fractions there is a band P, corresponding to fraction I of Baker et al. This once again testifies to the heterogeneity of substances forming a precipitation band corresponding to fraction I of Baker.

In figure 9 it is apparent that in the toxic fraction there is a large number of antigens since some lines are noticeably more intensive than with the initial extract of bacteria.

In conclusion it must be noted that the suggested lay-out for the arrangement of the antigens is true only for the strain of plague bacillus being cultivated (P. pestis EV (NIIEG)) which was cultivated on a medium of caseinic acid hydrolysate at 37°. Other strains of plague bacillus may have other concentrations of antigens which will strongly influence the relative arrangement of the precipitation bands. The absence of certain antigens or the presence of others will change the general picture. Therefore for the reliable identification of

these antigens it is necessary to isolate them in pure form, prepare monovalent antisera, and by using these preparations set about interpreting the antigenic structure of avirulent and virulent strains of the plague bacillus.

We adhere to the opinion that the modified method of double diffusion precipitation in agar which we adopted (method B) makes it possible to better detect the presence of existing antigens in an extract of a bacterial mass and doesn't permit the emergence of Liesegang's phenomena. The recent investigations of Allison and Humphrey (1960), conducted with normal and labeled antigens, yet again testify to the fact that the corresponding antigens do not penetrate the zone of visible precipitation and only a negligible amount of antibodies pass through. The illustrations presented by Oudin (1952) and also by other investigators of the formation of several zones of precipitation with the periodic supplementing of the receptacles with reacting substances are in our opinion the result of the appearance of several protein components in the antigens used (mistakenly taken as homogeneous proteins). Thus, for example, chemical analysis showed that egg albumen is a mixture of several proteins (Rhodes, Azari and Feeney, 1958), and naturally a preparation of this substance could yield several precipitation zones when utilizing a method which possesses a great resolving power.

Conclusions

1. A scheme has been proposed for the distribution of 28 precipitation zones, caused by antigenic substances of the plague bacillus (P. pestis EV NIIEG) cultivated on a medium of caseinic acid hydrolysate.

2. The distribution has been studied of the antigens in fractions from an extract of plague bacillus which were obtained by separation with ammonium sulfate.

3. The heterogeneity was shown of fraction I of Baker et al. which consists of at least 6 components.

4. The purity was confirmed of fractions IA and IB isolated according to the scheme of Baker et al. from an admixture of other antigens.

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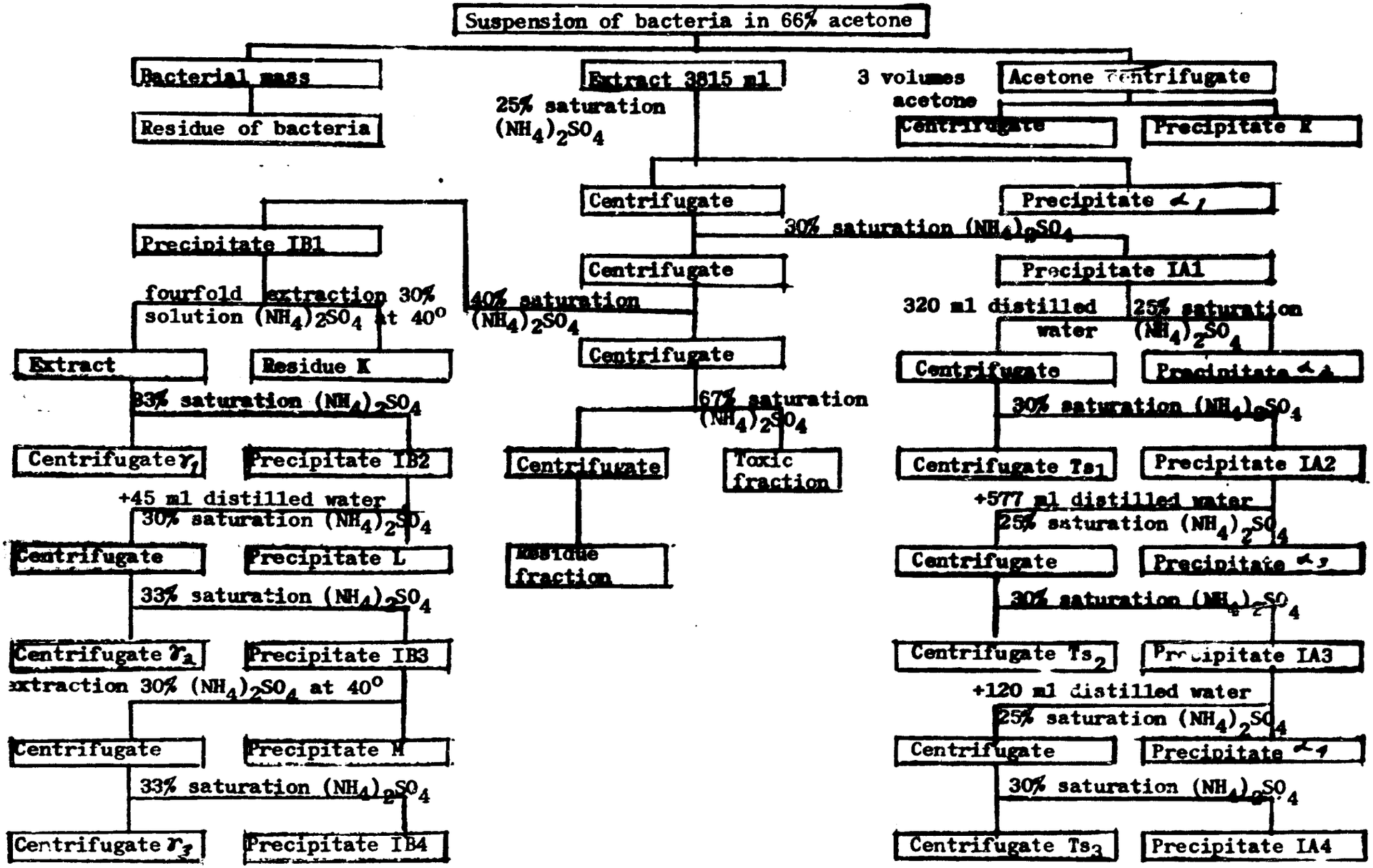

Fig. 1. Fractionation of the antigenic substances of an extract of P. pestis EV (NIIEG).

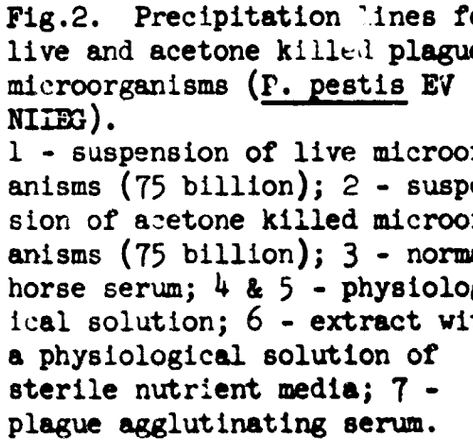

Fig.2. Precipitation lines for live and acetone killed plague microorganisms (P. pestis EV NIIEG).
1 - suspension of live microorganisms (75 billion); 2 - suspension of acetone killed microorganisms (75 billion); 3 - normal horse serum; 4 & 5 - physiological solution; 6 - extract with a physiological solution of sterile nutrient media; 7 - plague agglutinating serum.

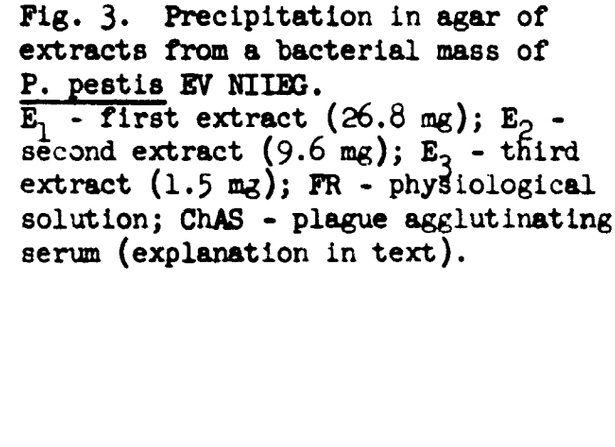

Fig. 3. Precipitation in agar of extracts from a bacterial mass of P. pestis EV NIIEG.
E_1 - first extract (26.8 mg); E_2 - second extract (9.6 mg); E_3 - third extract (1.5 mg); FR - physiological solution; ChAS - plague agglutinating serum (explanation in text).

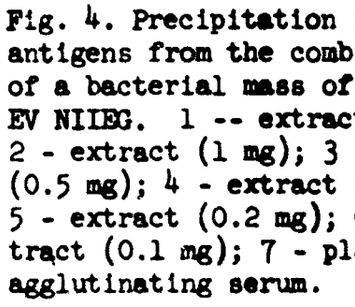

Fig. 4. Precipitation in agar of antigens from the combined extract of a bacterial mass of P. pestis EV NIIEG. 1 -- extract (5 mg); 2 - extract (1 mg); 3 - extract (0.5 mg); 4 - extract (0.3 mg); 5 - extract (0.2 mg); 6 - extract (0.1 mg); 7 - plague agglutinating serum.

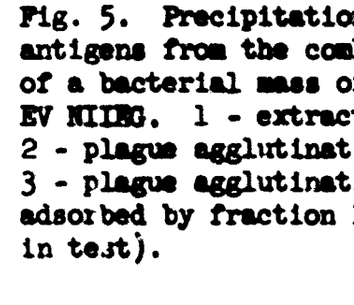

Fig. 5. Precipitation in agar of antigens from the combined extract of a bacterial mass of P. pestis EV NIIEG. 1 - extract (15 mg); 2 - plague agglutinating serum; 3 - plague agglutinating serum adsorbed by fraction IA (explanation in text).

Fig. 6. Precipitation in agar of the antigens from a combined extract of a bacterial mass of P. pestis EV NIIEG (explanation in text).
1 - extract (15 mg); 2 - plague agglutinating serum; 3 - plague agglutinating serum absorbed by fraction IA.

Fig. 7. Schematic of the arrangement and designation of the antigens of P. pestis EV NIIEG.

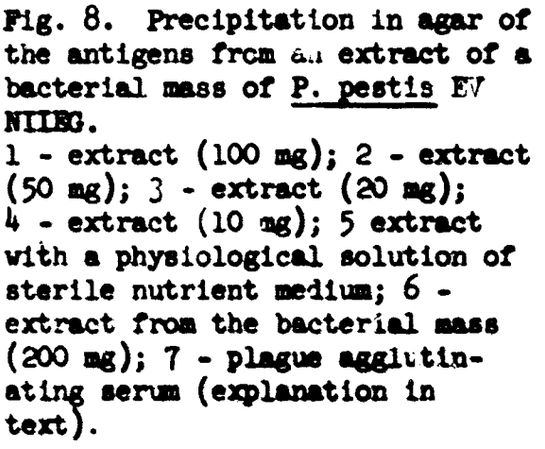

Fig. 8. Precipitation in agar of the antigens from an extract of a bacterial mass of P. pestis EV NIIEG.
1 - extract (100 mg); 2 - extract (50 mg); 3 - extract (20 mg); 4 - extract (10 mg); 5 extract with a physiological solution of sterile nutrient medium; 6 - extract from the bacterial mass (200 mg); 7 - plague agglutinating serum (explanation in text).

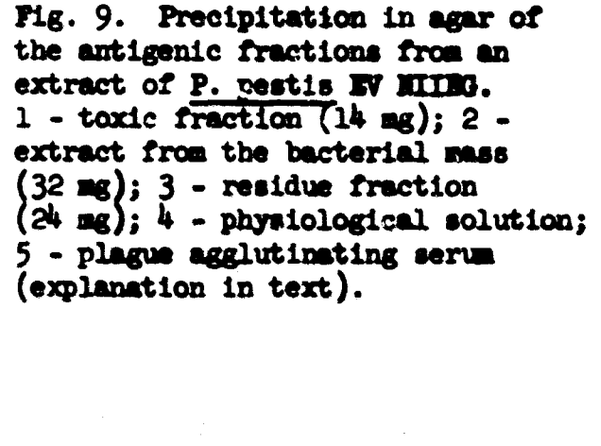

Fig. 9. Precipitation in agar of the antigenic fractions from an extract of P. pestis EV NIIEG.
1 - toxic fraction (14 mg); 2 - extract from the bacterial mass (32 mg); 3 - residue fraction (24 mg); 4 - physiological solution; 5 - plague agglutinating serum (explanation in text).